Spatial Profiling of the Head and Neck Squamous Cell Carcinoma Microenvironment: Reshaping Our Understanding and Therapeutic Opportunities

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Abstract:

Head and neck squamous cell carcinoma (HNSCC) is characterized by a highly heterogeneous tumor microenvironment (TME) that drives disease progression and therapeutic resistance. The emergence of spatial omics technology, encompassing spatial transcriptomics, proteomics, metabolomics and multi-modal imaging, has revolutionized our understanding of TME by preserving the critical spatial architecture of molecular landscapes. This review synthesizes key innovations derived from spatial omics in HNSCC TME, detailing the organised spatial distribution of malignant cell states, the functional organization of immune cells (e.g., tertiary lymphoid structures vs. suppressive stromal niches), and the pivotal roles of heterogeneous cancer-associated fibroblasts (CAFs) and abnormal vasculature. We further decipher spatially resolved intercellular communication networks that mediate drug resistance mechanisms to immunotherapy, and targeted therapy. Finally, we address current methodological constraints and discuss the transformative potential of integrating artificial intelligence with spatial data to refine patient stratification and personalize therapeutic strategies.

Keywords: head and neck squamous cell carcinoma; tumor microenvironment; multi-Omics technologies; cancer-associated fibroblasts; intercellular communication networks.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is a group of malignant tumors with strong molecular heterogeneity and high invasiveness, originating from the oral cavity, pharynx, larynx, and other sites within the head and neck region (1). The disease is characterized by persistently high rates of locoregional recurrence or distant metastasis, and the long-term survival rate is still stagnant despite the adoption of multi-modal treatment (2-4). More and more evidence shows that the tumor microenvironment (TME)-the diverse cellular and non-cellular components surrounding the tumor, is a key regulatory factor in the pathogenesis of the disease, and its composition and functional status directly affect the tumor progression, immune escape mechanism and the final treatment response (5-7). The HNSCC TME constitutes a highly dynamic, multicellular ecosystem comprising malignant epithelial cells, heterogeneous immune cell populations, cancer-associated fibroblasts (CAF) -key supportive cells in the tumor environment, vascular networks, and various extracellular matrix (ECM)-the complex network of non-cellular molecules providing structural and biochemical support to cells-components, which collectively exhibit notable inter- and intratumoral heterogeneity (8-10).

The past decade has witnessed a revolution in our understanding of TME heterogeneity with the advent of spatial omics technologies. These powerful tools, including spatial transcriptomics (Xenium (10x Genomics), CosMx SMI (Nanostring, now part of Bruker), RNA scope), spatial proteomics (GeoMx DSP, Imaging Mass Cytometry (IMC), Deep visual proteomics), multiplexed imaging (PhenoCycler-Fusion (PCF, formerly CODEX, from Akoya Biosciences-a Quanterix company), Multiplex Immunofluorescence (mIF)), and emerging spatial metabolomics, have revolutionized our ability to study TME heterogeneity by analysing molecular characteristics at a resolution close to single cells while retaining the original structure of the tissue (11-15). As shown in **Table 1**, each technology class has

unique advantages: the transcriptomics platform reveals the gene expression process; the proteomics method characterizes functional effect proteins; metabolomics imaging captures the distribution of small molecules; and the emerging epigenomics technology draws regulatory maps. This progress can accurately locate cell subtypes, like various malignant cells, immune cells and stromal cells; and reveal how these cells are organized in different functional microenvironments, such as immune "cold zones" or hypoxic regions (16-18). Unlike traditional single-cell approaches, spatial omics provides critical insights into the spatial relationships and interactions among different cell subtypes, which are essential for understanding tumor progression, immune evasion, and therapy resistance (19). The integration of spatial and molecular data enables us to reconstruct the intercellular communication network in the tumor, so as to fully understand the dynamic tumor ecosystem.

In the context of HNSCC, spatial omics provides unprecedented opportunities for decoding the spatial drivers of tumor heterogeneity, identifying recurrence-related microenvironments, and developing spatial information biomarkers for clinical stratification. This review will focus on the innovative application of spatial technology in revealing HNSCC TME, especially on: (i) drawing cell heterogeneity maps within the spatial framework; (ii) clarifying intercellular communication patterns and microenvironment formation; and (iii) transforming spatial information into individual precision treatment strategy. By promoting the integration between multi-group research, computational biology and clinical oncology, spatial oncology is expected to transform precision oncology from molecular spectrum analysis to space-guided intervention treatment.

Table 1: Overview of Spatial Multi-Omics Technologies

Omics Layer	**Core Technology/**	**Molecules Detected**	**Spatial Resolution**	**Key Advantages**	**Major Challenges**

	Platforms				
Spatial Transcriptomics	Visium/Xenium (10x Genomics), CosMx SMI (NanoString), BGI Stereo-seq, MERFISH, RNAscope	Whole transcriptome or targeted RNAs (100s-1000s)	Spot-level (~55 μm) to subcellular	Unbiased discovery of gene expression programs and cell states	Lacks protein information, cell phenotype often requires inference
Spatial Proteomics	IMC, PCF, GeoMx DSP, Deep visual proteomics,mIF, AKOYA PCF	Proteins (10s-100s)	Single-cell to subcellular	Direct detection of functional effectors, strong clinical pathology link	Limited by antibody availability, unable to discover novel targets
Spatial Metabolomics	MALDI-MSI, DESI-MSI	Metabolites, lipids, drugs (100s)	~10-50 μm	Label-free detection of functional phenotypes	Difficult metabolite identification, incomplete databases
Spatial Epigenomics	Spatial-ATAC-seq	Chromatin accessibility, histone modifications	Currently low, improving	Reveals regulatory mechanisms of gene expression	Technically immature, fewer applications

Integrated Multi-Omics	CosMx, GeoMx	RNA and protein simultaneously	Platform-dependent	Direct correlation of different molecular layers in same spatial context	Complex experimental design, high computational demand for integration

2. An Overview of Spatial Omics Technologies

Spatial omics covers a series of rapidly developing technologies that can comprehensively analyze molecular information (including RNA, protein and metabolite abundance) in the context of the complete structure of tissue slices (20-22). According to their basic principles, these methods can be roughly divided into two categories: imaging-based methods and sequencing-based methods (23, 24).

Imaging-based technologies, such as mIF, IMC and PCF, use antibody or oligonucleotide-labeled probes to visualize dozens to hundreds of proteins or RNA at the same time with subcellular resolution (25-27). These platforms excel at characterizing cell phenotypes and states with high spatial fidelity but are limited by pre-defined targets. In contrast, sequencing-based technologies, such as 10x Genomics Visium/Xenium, CosMx SMI and stereo sequencing, either capture polyadenylated RNA (poly(A) RNA) upregulate(Visium) from the spatial barcode region, or directly image and quantify hundreds to thousands of RNA transcripts (Xenium, CosMx SMI, MERFISH) at single cell resolution (28, 29). These methods offer a more unbiased discovery potential for novel gene expression patterns but may have varying resolution and throughput (28, 29).

Since these platforms have their own advantages and disadvantages, choosing the appropriate technology requires clear experimental goals **(Table 2).** Technical choices

usually require a trade-off between resolution, multiple analysis ability, sample flux and discovery ability. It is crucial to combine these spatial data with complementary models (such as single-cell RNA sequencing), which can be achieved by calculating "anti-convolution" or "integration" methods, which are crucial for inferring cell type composition and in-depth understanding of the system-level mechanism of cell ecosystems (16, 30).This comprehensive technical foundation, leveraging the unique capabilities of various spatial omics platforms in terms of resolution and multiplexing, enables researchers to go beyond a simple list of cell diversity. It provides unprecedented opportunities to precisely map the cellular composition and heterogeneity within HNSCC tumor microenvironments in their native tissue context, thereby actively decoding the spatial laws that govern these complex ecosystems.

Table 2. Comparison of Major Spatial Omics Platforms on Sequencing- and Imaging-Based Levels.

Sequencing-Based	**10x Visium**	**10x Xenium**	**10x Visium HD**	**CosMx SMI**	**Stereo-seq**
Resolution	Low (~55-100 μm spots)	Subcellular (~200 nm)	High (near single cell)	Subcellular	500 or 715 nm
Throughput	Whole transcriptome	Targeted panels (100s of genes)	Whole transcriptome	1000+ RNAs, 64+ proteins	Genome-wide
Panel Type	Whole transcriptome	Fixed/custom RNA probes	Whole transcriptome	Targeted RNA/Protein	Whole transcriptome

Strengths	Broad transcriptome survey	High-res cell-level biology, rare cell types	High-res transcriptomics + full gene coverage	Ultra-high-plex, multi-omics	Extremely high resolution & large field of view, unbiased
Limitations	Not single-cell, requires deconvolution	Targeted panel, higher cost	Larger data size, higher cost	Complex platform	Requires specialized bioinformatics expertise
Ideal for	General tissue-level trends	Cell interactions, FFPE pathology, defined gene sets	Cell-level spatial studies with full gene coverage	High-plex, single-cell resolution mapping of both RNA and protein targets	comprehensive, cell-level maps of large tissue areas,
Imaging-Based	**CODEX**	**NanoString GeoMx DSP**	**mIF**	**MERFISH**	**seqFISH/+**
Resolution	Subcellular	50-100 µm (Region of Interest)	Single-cell	Subcellular	Subcellular
Throughput	40-60 proteins	Whole Transcriptome/100+ proteins	6-10 markers	2D: 100–1,000 genes	10,000+ genes

				3D: 10,000 genes	
Panel Type	Protein	RNA/Protein	Protein	Targeted RNA	Targeted RNA
Strengths	Extremely high resolution, cell phenotyping	Region-of-interest flexibility, high-plex	High clinical adoption, easy integration	Extremely high sensitivity and resolution for RNA	Highest multiplexing capacity for imaging RNAs
Limitations	Antibody-dependent, complex tissue processing	ROI selection can be subjective, not single-cell	Limited plex, channel crosstalk	Requires specialized imaging, complex probe design	Long imaging times, complex data analysis
Ideal for	High-dimensional, single-cell phenotyping of protein expression	Hypothesis-driven, high-plex spatial profiling of predefined tissue regions	Validating established cellular biomarkers and spatial relationships	Mapping the precise subcellular localization of hundreds to thousands of RNA transcripts.	Near-complete transcriptome imaging at subcellular resolution.

3. Cellular Composition and Spatial Heterogeneity in HNSCC TME: Insights from Spatial Omics

Building upon the advanced capabilities of spatial omics technologies discussed in the previous section, we can now gain unprecedented insights into the intricate cellular composition and spatial heterogeneity of the HNSCC TME.

3.1 Malignant Cell Subtypes and Their Spatial Distribution

As revealed by these high-resolution approaches, HNSCC exhibits significant heterogeneity at the cellular level, encompassing both molecular and spatial dimensions (31). For instance, the malignant epithelial cells in HNSCC do not exist as discrete subtypes but typically present a series of phenotypic states, mainly along the gradient of epithelial-mesenchymal transition (EMT) (9, 10). Spatial omics has been instrumental in demonstrating that the dominant cell populations usually retain the core epithelial phenotype, characterized by strong intercellular adhesion, high proliferative activity, and being mainly located in the tumor core (TC) (32). Conversely, cells that simultaneously express epithelial and mesenchymal markers and have partial EMT (pEMT) features are often enriched at the invasive margins (33). This precise spatial distribution, elucidated by spatial technologies, suggests that pEMT plays a direct role in promoting local tissue invasion and spread. Moreover, malignant cells with cancer stem cell-like characteristics (typically associated with the EMT process and resistant to treatment) are not randomly distributed (34). They tend to cluster in specific microenvironments, such as around blood vessels or in hypoxic regions, where spatial interactions with stromal cells support their maintenance (35, 36). Therefore, the spatial structure of HNSCC, from the proliferation core to the invasion edge containing pEMT and stem cell-like cells, reflects a functional tissue that is the basis for tumor progression and treatment resistance.

3.2 Immune Cell Populations and Spatial Organization

The spatial structure of the immune microenvironment is the basic regulating factor for the immune and therapeutic response of tumors in squamous cell carcinoma of the head and neck, and its role goes far beyond the existence of immune cells. As highlighted by *Fu et al*., the formation of tertiary lymphoid structures (TLS) at the tumor-stroma interface is a key structural feature that promotes the synergistic activation of T cells and B cells, and its existence is closely related to better patient prognosis (37). *Li et al.* further clarified the functional importance of TLS; their findings suggested that $CD4^+$ T cells depleted by progenitor cells in mature TLS may act as core regulatory factors that activate and maintain the response of T cells and B cells in tumors, thus providing a potential mechanistic basis for their clinical benefits (38). Nevertheless, such anti-tumor immunity is often counteracted by spatially organized immunosuppressive mechanisms. However, this anti-tumor immunity is often antagonized by the immunosuppressive mechanism of spatial organization. In HNSCC, immune escape usually stems from spatial antagonism, that is, the function of $CD8^+$ T cells is inhibited through regulatory interactions with cancer-related fibroblasts (39, 40). This suppression is further exacerbated by the increase in the spatial proportion of regulatory T cells (Tregs) to $CD8^+$ T cells, as well as the presence of $CD56^{dim}$ NK cells and M2-polarized macrophages, all of which are gathered in a specific structural microenvironment, thus destroying effective immune targeting (5, 41). Importantly, this immune microenvironment is not fixed, but a significant spatial evolution occurs when the disease recurs, which seriously weakens the therapeutic effect. *Watermann et al.* confirmed that recurrent HNSCC showed significant changes in the tumor immune microenvironment (TIME), which was characterized by a significant decrease in $CD8^+$ T cells and B lymphocytes, as well as the relative amplification of neutrophils and macrophages (42). In a word, deciphering the spatial logic of immune interaction (including the position of inhibiting cells relative to cytotoxic effects and the physical barrier that restricts T cell infiltration) is crucial to the development

of spatial information immunotherapy that can successfully reprogram the immunosuppressive HNSCC microenvironment.

3.3 Stromal Components and Their Spatial Niches

In addition to the immune components, the stromal components, especially CAFs, are the main builders of the TME in HNSCC and play a crucial role in the physical structure and functional state of the tumor (43). CAFs are not a single cell population but exhibit significant heterogeneity. Different subtypes occupy specific spatial ecological niches and drive specific aspects of tumor biology (44). In HNSCC, several key CAF subpopulations have been identified: myofibroblastic CAFs (myCAFs), which are usually located in the TC and help form a dense and rigid ECM; inflammatory CAFs (iCAFs), which are usually located in the tumor periphery and secrete various cytokines and growth factors to promote inflammation and angiogenesis; and antigen-presenting CAFs (apCAFs), which can directly interact with immune cells (45). This article comprehensively outlines the spatial and functional heterogeneity of these CAF subpopulations, which is a key resource for understanding their unique contributions to the TME **(Table 3)**.

Other than the above basic classifications, emerging studies in spatial cancer research have begun to clarify the exact role of specific CAF subtypes in the progression of HNSCC. For instance, *Liu et al.* demonstrated through the integration of single-cell and spatial transcriptomic data that POSTN+ CAFs promote cancer cell metastasis through EMT, thereby facilitating lymph node metastasis (LNM) in oral squamous cell carcinoma (OSCC, the main subtype of HNSCC) (16). In another spatially resolved study, *Li et al.* identified *IFN-induced MHC-IhiGal9*$^{+}$CAFs, which form an immunosuppressive microenvironment that effectively captures CD8+ T cells and weakens their cytotoxic function in the TME (40). *Wang et al.* further strengthened the clinical significance of specific CAF subgroups; they found that *SFRP2*$^{+}$CAFs were associated with enhanced tumor

development and the poor survival prognosis of HNSCC patients (46). Overall, these findings emphasize the crucial role of spatially distinct CAF subtypes in driving disease invasion and immune escape. Therefore, analyzing the spatial and functional specialization of CAF subgroups - including their coordinated microenvironments (which regulate immunosuppression, extracellular matrix remodeling, and metastasis and spread) - is crucial for developing new therapies that can successfully dismantle these pathological ecosystems in HNSCC.

Table 3. Key CAF Subtypes and Their Functions in HNSCC

CAF Subtype	Characteristic Markers	Spatial Localization	Key Functions	Implication for Therapy
myCAF	α-SMA, FAP	Tumor Core	Deposits dense ECM, forms a physical barrier	Impedes drug penetration, chemoresistance
iCAF	IL-6, IL-11, CXCL12	Peri-tumoral/ Stromal Regions	Secretes inflammatory cytokines, recruits immunosuppressive cells	Drives T-cell exhaustion, immunotherapy resistance
apCAF	MHC-II, CD74	Immune-Rich Niches	May present antigen to CD4+ T cells; context-dependent role	Complex, potentially immunosuppressive
***POSTN*$^{+}$ CAF**	Periostin (POSTN)	Invasive Front	Promotes EMT, creates a pro-metastatic niche	Associated with lymph node metastasis, poor prognosis
MHC-IhiGal9^{+} CAF	MHC-I, Galectin-9	Immunosuppressive Niche	Sequesters and disables CD8+ T cells via Galectin-9	Contributes to non-response to immunotherapy

FRC-like CAF	CCL19, CCL21	Within TLS	Supports TLS structure and function, coordinates adaptive immunity	Associated with response to immunotherapy and favorable prognosis

3.4 Vascular and Lymphatic Endothelial Cells in the TME

In HNSCC, blood vessels and lymphatic vessels are active regulators of the tumor microenvironment, which play a crucial role in immunosuppression dynamics and metastasis (47, 48). Tumor-associated endothelial cells show unique functional phenotypes, which are characterized by changes in the expression of adhesion molecules, growth factor receptors and immunomodulatory ligands (49). Through the upregulated secretion of factors such as vascular endothelial growth factor A (VEGFA) and CXCL12, they drive the formation of vascular disorders and high permeability, which not only forms a hypoxic microenvironment, but also forms physical barriers and chemokine-mediated barriers to limit the infiltration of $CD8^+$ T cells (50, 51). Evidence suggests that lymphatic endothelial cells may also contribute to activated T cell apoptosis by upregulating PD-L1 and presenting FAS ligands, potentially promoting the immune escape of tumor cells; at the same time, their strategic positioning at the edge of invasion further promotes the spread of tumor cells to regional lymph nodes (52, 53). Recent single-cell and spatial transcriptomic studies have further revealed that there is significant heterogeneity between these endothelial cells, and identified specialised subgroups with different transcriptional procedures, which support specific processes such as angiogenesis, immunomodulation and treatment of drug resistance (54). In short, these findings transform endothelial cells from passive channels into dynamic regulators of tumor progression, emphasizing the importance of vascular normalization and targeted inhibition of specific endothelial immune checkpoints as potential treatment strategies for HNSCC.

The studies detailed in this section together show that the TME of HNSCC is not a mixture of cells, but a highly ordered ecosystem composed of specialised functional ecological sites. In order to synthesise these spatial relationships, we proposed a conceptual model that maps key cell components to their unique topological environment **(Figure 1)**. This figure visually maps the key cell components—including malignant cells (tumor cells, cancer stem cells), immune populations (T cells, B cells, macrophages), cancer-associated fibroblasts, and associated vasculature (blood cells)—to their unique topological environments within the HNSCC TME. This comprehensive spatial framework, which integrates the diverse elements discussed in Sections 3.1-3.4, lays the necessary foundation for understanding the intercellular communication network to be discussed in the next section.

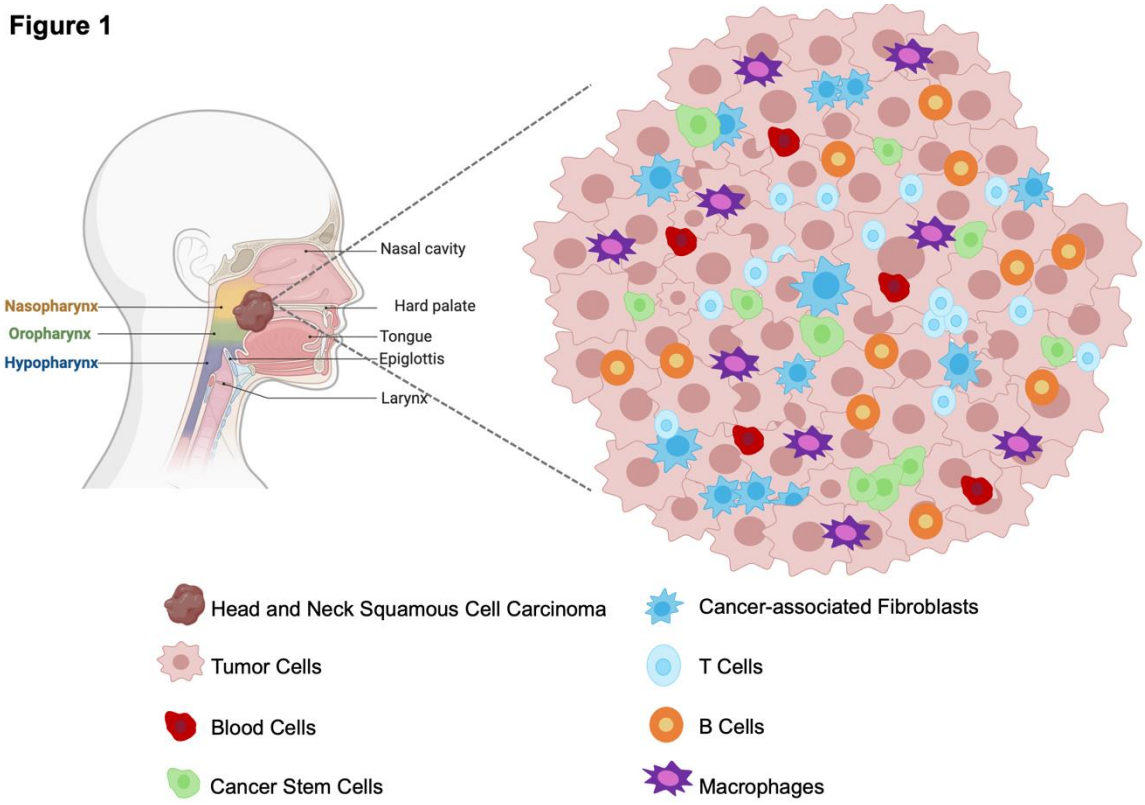

Figure 1. The Spatial Architecture HNSCC TME. This schematic provides a conceptual overview of the HNSCC TME, illustrating the distinct spatial localization and interactions of various cellular components. The left panel exhibits the common anatomical locations where HNSCC tumors typically arise within the human head and neck region (e.g., nasopharynx,

oropharynx, hypopharynx, tongue, larynx), with the dark brown mass representing a macroscopic HNSCC tumor. The right panel provides a conceptual schematic of the HNSCC TME at a microscopic level, showcasing the diverse cellular components that interact within this complex ecosystem. It depicts Tumor Cells (pink, spiky outline) as the predominant malignant population, intermingled with other critical cell types including Cancer Stem Cells (green), Blood Cells (red), Cancer-associated Fibroblasts (light blue stars), T Cells (light blue circles), B Cells (orange circles), and Macrophages (purple stars). This schematic emphasizes the cellular diversity and the general mixed distribution of components within the HNSCC TME.

4. Spatially Resolved Cell–Cell Crosstalk in HNSCC

4.1 The Spatially Organised Immune Landscape: Beyond Cold and Hot

Current spatial studies of HNSCC have improved the classic "hot" and "cold" immune classification, revealing that the immune phenotype depends not only on the density of immune cells, but also on the coordinated spatial interaction between immune cells and specific stromal components (55, 56). The "hot" region represented by HPV-positive tumors is characterized by the existence of a structured TLS; crucially, these microenvironments are rich in *CCL19*$^{+}$ fibroblasts, which are spatially associated with CD4+ T cells and B cells, thus supporting the coordinated anti-tumor immune response and are related to the improvement of the efficacy of immunotherapy (56). On the contrary, the "cold" region, more common in HPV-negative tumors, is characterized by the spatial rejection of cytotoxic T cells. This immunosuppressive environment is mainly composed of iCAF, myCAF and proto-CAF (low expression level of myCAF/iCAF marker gene in CAF cluster). They are spatially associated with inflammatory monocytes and help form a matrix barrier, thus hindering the infiltration and function of effective T cells (56). Therefore, the precise topology of these

different CAF subtypes is the fundamental determinant of the HNSCC immune microenvironment and its clinical impact.

4.2 Stromal-Immune Interactions: Orchestrating Suppression and Exclusion

In HNSCC, CAFs actively shape the immunosuppressive microenvironment through various spatial recognition mechanisms. Specific CAF subtypes play a particular role: *POSTN*+ CAFs at the invasive margin form physical ECM barriers, excluding CD8+ T cells from the tumor nest; *MHC-IhiGal9*$^{+}$ CAFs form de facto traps, through ligand-receptor interaction, isolating and inhibiting the function of T cells; while *CXCL13*$^{+}$ CAF promote B cell adhesion and antibody production, activating CXCL13^{+}CD8^{+} T cells that become exhausted in tumor cell aggregates in nasopharyngeal carcinoma (a kind of HNSCC) (16, 40, 57). In addition to physical exclusion, apCAFs may also promote the formation of immunosuppressive microenvironments by regulating the composition of local T cells, which may promote the advantages of CD4^{+} T cells over CD8^{+} T cells, and the advantages of CD4^{+} T cells are related to tumor progression (58). Simultaneously, iCAFs secrete soluble factors such as *CXCL12* and *TGF-β*, establishing chemokine gradients, recruiting regulatory T cells and promote T-cell exhaustion in peri-tumoral regions (59). Through these various spatial interactions, CAFs become the core regulatory factors of the immunosuppressive stroma in HNSCC. Spatially resolved identification of these distinct CAF populations and their interactions with immune cells is critical. For instance, if spatial data reveals an abundance of POSTN^{+} CAFs physically excluding T cells, it directly suggests a treatment strategy combining immune checkpoint inhibitors (ICIs) with CAF-targeting agents (e.g., FAP inhibitors) or ECM-modifying drugs to overcome the physical barrier and enhance T cell infiltration and function.

4.3 Vascular and Hypoxic Niches: Hubs for Immune Evasion and Therapy Resistance

The abnormal vascular system and hypoxia caused by HNSCC form a unique spatial microenvironment, which is crucial for immune escape and drug resistance treatment (60). Abnormal, leaky blood vessels, driven by high VEGFA signaling will not only form a hypoxic and acidic microenvironment, which directly inhibits the function of T cells, but also cannot effectively support the infiltration of T cells, thus forming a physical barrier that hinders infiltration (61). In these hypoxic microenvironments around the blood vessels, tumor cells and stromal cells will upregulate immunosuppressive metabolites, such as immune checkpoints, further inhibit the activity of local T cells, and promote the microenvironment conducive to the survival of drug-resistant stem cell-like cancer cells (41, 50). Spatial profiling technologies are instrumental in precisely mapping these hypoxic niches and abnormal vascular structures. Identifying such regions within a tumor can directly inform treatment decisions, suggesting combination therapies that include anti-angiogenic agents or hypoxia-modifying drugs alongside immunotherapies to improve oxygenation, normalize vasculature, and enhance immune cell access and function.

4.4 Reconstruction of Global Communication Networks from Spatial Data

The real advantage of spatial biology is that it can directly reconstruct and quantify the intercellular communication network in the tissue structure (62). By analyzing the co-localization of ligands and receptor mRNA or proteins, key signaling pathways can be mapped to specific cell regions (63-65). For instance, spatial transcriptomics has revealed that in glucose-deficient regions of HNSCC, cancer cell-derived CXCL8 interacts with macrophages to establish a feedforward loop that promotes antioxidant production and confers resistance to nutrient-starvation therapies (anlotinib) (63). In HPV-negative HNSCC, systematic profiling of ligand-receptor interactions has identified prognostic signaling networks involving ECM and immune regulation, whose integration with histopathological features enables improved risk stratification (64). In addition, spatial analysis revealed that

there is a mutually exclusive expression pattern between the new immune checkpoint B7-H4 (VTCN1) and PD-L1. The B7-H4-positive tumor region shows significant rejection of $CD8^+$ T cells, highlighting its potential as an immune cold tumor treatment target (65). Integrating these spatially resolved interactions into global network models not only identifies dominant signaling circuits driving tumor progression but also nominates novel targets for disrupting the pathological ecosystem of HNSCC.

5. Spatial Features Linked to Therapy Response and Resistance in HNSCC

5.1 Immunotherapy

Immune checkpoint inhibitors (ICIs), especially PD-1/PD-L1 blockers, have significantly reshaped the treatment pattern of some patients with HNSCC, but the overall response rate is still not high (66-68). Spatial omics plays an important role in revealing the efficacy of ICIs. Its efficacy depends not only on the existence of CD8+ T cells, but also on the integrated spatial tissue structure of various immune cell types in the TME (69). This refined understanding provided by spatial data directly impacts patient stratification and treatment planning. For instance, the spatial identification of mature, functional TLS within tumors, rich in CD4+ T cells, memory B cells and plasma cells, serve as a robust positive spatial predictive biomarker for better response to ICIs (70). Patients whose tumors exhibit such organized TLS, detectable through spatial profiling, could be prioritized for immunotherapy or considered for de-escalation strategies if response is robust. On the contrary, the spatial structure related to ICI drug resistance is usually manifested as CD8+ T cells closely adjacent to immunosuppressive components (such as Treg) in the perivascular microenvironment or M2-like macrophages in the stromal region) (71). In these cases, spatial analysis directly informs the need for combination strategies, such as adding agents that deplete suppressive cells (e.g., anti-CCL22 to target Tregs) or disrupt physical barriers (e.g., targeting specific

CAFs), to reprogram the TME and improve ICI effectiveness. This spatially informed approach moves beyond simple PD-L1 expression or CD8+ T cell density, allowing for a more precise selection of patients who will truly benefit from ICIs and guiding the design of rational combination therapies for non-responders. In addition, B cells and plasma cells located outside the TLS structure may also acquire tumor-promoting properties, which further illustrates how the spatial environment determines immune function (57). In short, the response of HNSCC to immune checkpoint inhibitors reflects a balance between effective immune activation in the TLS structure and functional inhibition in the stromal or vascular microenvironment - this balance is spatially regulated.

5.2 Targeted Therapies

TP53 represents a frequently common mutation gene in HNSCC, often with a high mutation rate around 70%, which can vary significantly depending on factors such as HPV status and anatomical subsite (72, 73). In addition, the *EGFR* gene is widely altered, with up to 80–90% of HNSCCs exhibiting either overexpression or mutations (74). Other important mutations include *FAT1*, *PIK3CA*, *CDKN2A*, and *NOTCH1*, which together reveal the great potential of the treatment strategy (75-78). Despite this, at the molecular level, the clinical efficacy of targeted drugs is still unsatisfactory, highlighting the key transformation gap between genomic change and treatment success (79, 80). Spatial technology is revealing that the therapeutic response depends not only on the mutation state, but also on the spatial structure of target gene expression and the protective ecosystem in the tumor microenvironment (32, 39). For instance, spatial transcriptomics analysis of early-onset tongue cancer uncovers both significantly enriched MAPK and JAK-STAT signaling pathways and a distinctive TME characterized by increased plasma cell gene signatures and TLS featuring plasma cell and lymphocyte aggregations, particularly at the invasive front, thereby suggesting the need for customized targeted interventions for these specific

molecular subtypes (81). Similarly, studies of tumor budding have identified *NSD1* mutations as negatively correlated with budding and uncovered a critical role for CAV1 and MMP14 proteins. The expression gradient of these proteins from the tumor body to the budding highlights the spatially increasing invasion procedures, which may become therapeutic targets (82). Further spatial dissection of the tumor leading edge consistently identifies upregulated collagen, CD99, and non-canonical WNT signaling pathways that drive invasion, while the tumor core is characterized by Angiopoietin-like protein (ANGPTL) and POSTN cell signaling modules, which proposes a compartment-specific targeting strategy that emcompass both the tumor nest and the surrounding stroma (83). In precancerous lesions, the spatial conversion regulation of VEGF signals is combined with immunosuppressive mononuclear cells to create a microenvironment conducive to malignant transformation, which suggests a new blocking target (84). The most notable thing is that the integrated multi-omics analysis links POSTN-mediated extracellular matrix remodeling and CAF-secreted TGF-β with epithelial EMT and LNM in OSCC, which directly indicates that the POSTN-TGF-β axis is a potential target for breaking the transfer cascade reaction (16). In summary, these spatial discrimination insights go beyond the static mutation list and reveal the geographical distribution of targetable pathways and matrix-tumor interactions, thus providing a roadmap for the development of mechanism-based combined therapies and targeted treatment of head and neck squamous cell carcinoma (HNSCC) for specific situations.

5.3 Translational Outlook: Spatial Biomarkers for Clinical Stratification

The clinical transformation of spatial genomic research results requires the distillation of complex spatial data into operable biomarkers to guide patient selection, individualised treatment and real-time monitoring. Characteristics such as cell subtype, intercellular interaction, ligand-receptor analysis and spatial co-localization of tumor CAFs are potential

space biomarker candidates (85, 86). Specifically, spatial biomarkers derived from immune escape mechanisms, such as the spatial density and precise localization of specific CAF subtypes (e.g., $POSTN^+$ CAFs physically excluding T cells), the presence and maturity of TLS (indicating immune competence), or the spatial proximity of $CD8^+$ T cells to immunosuppressive myeloid cells (indicating resistance), can serve as powerful tools for patient stratification. These biomarkers can predict response to ICIs, identify patients who require combination therapies, and guide the selection of appropriate therapeutic partners to overcome specific spatially-driven immune escape mechanisms.

Combined with digital pathology and computer image analysis, automatic quantitative analysis of these characteristics in conventional histological specimens can be realized, thus promoting their scalable application in clinical workflow (87-89). For example, deep learning models extracting spatial features—such as tumor infiltrating lymphocyte density, tumor microenvironment heterogeneity, and granulocyte enrichment at the invasive margin—from standard H&E slides have demonstrated a remarkable ability to predict overall survival benefit from the PI3K inhibitor buparlisib in recurrent/metastatic HNSCC, even outperforming conventional CD3 immunohistochemistry (89). Digital pathology platforms combined with computational analysis are being utilized to standardize challenging PD-L1 combined positive scoring by objectively quantifying staining intensity and distribution, thereby reducing inter-observer variability and technical discordance between different assay platforms (90). In addition, a computational pipeline applied to digital pathology slides has successfully identified prognostically relevant spatial markers, such as the spatial distribution pattern of FOXP3 across tumor and stromal compartments, providing a cost-effective strategy for spatial biomarker discovery (91). In the context of immunotherapy, an AI-driven single-cell spatial biomarker quantifying specific cell-cell interactions within the tumor microenvironment has proven superior to PD-L1 expression alone in predicting progression-

free survival and objective response to immune checkpoint inhibitors in advanced non-small cell lung cancer, with potential applicability to HNSCC (87). These examples together highlight the transformation potential of combining computational pathology with spatial biology to generate robust, automated and clinically applicable biomarkers. Crucially, the validation of these spatial biomarkers in prospective, multi-center clinical trials is the next imperative step to translate these experimental findings into improved clinical outcomes for HNSCC patients.

Future multi-center clinical trials should include spatial biomarker endpoints to verify their predictive and prognostic efficacy, evaluate their repeatability on different platforms, and determine their cost-effectiveness. In the end, integrating spatial biology into clinical decision-making will bridge the gap between molecular spectrum analysis and microenvironment-based intervention, and provide a new paradigm for the individualized treatment of HNSCC.

6. Limitations

While spatial omics approaches offer unprecedented insights into the localized molecular intricacies of diseases, they are accompanied by several methodological and translational limitations that warrant consideration. A significant challenge lies in reproducibility and protocol standardization, as the diverse array of platforms and complex experimental workflows can lead to variability across studies and laboratories, hindering robust cross-study comparisons. Sample preparation remains critical; for instance, the use of formalin-fixed paraffin-embedded (FFPE) tissues, while abundant, can introduce technical biases due to RNA degradation and fragmentation, potentially impacting transcript capture efficiency and data quality. Conversely, fresh frozen samples, though providing superior RNA integrity, present their own challenges in tissue handling and morphological preservation. Furthermore,

current spatial transcriptomics technologies often operate at a resolution that is multi-cellular rather than true single-cell, which can obscure the precise attribution of gene expression to individual cell types within a heterogeneous TME. Technical biases can also arise from platform-specific capture efficiencies, probe design, and batch effects. Finally, the interpretation of these complex, high-dimensional spatial datasets demands sophisticated bioinformatics tools and expertise, posing a significant hurdle for many research groups. From a translational perspective, the high cost, relatively low throughput for large patient cohorts, and the current lack of standardized diagnostic pipelines mean that integrating these powerful spatial omics findings into routine clinical practice remains a substantial future challenge. Addressing these methodological gaps will be pivotal for transitioning spatial omics from a discovery tool to a routine clinical diagnostic platform.

7. Conclusion and Perspective

Spatial omics has transitioned HNSCC research from bulk-level averages to a high-resolution, context-dependent understanding of tumor biology. By mapping the precise coordinates of cellular interactions, these technologies have uncovered novel biomarkers and structural motifs—such as the invasive budding front and mature TLS—that hold significant prognostic value. However, the clinical translation of spatial oncology remains hindered by high costs, a lack of protocol standardization, and the immense complexity of data integration.

Looking forward, the convergence of spatial multi-omics with artificial intelligence and deep learning will be pivotal. AI-driven platforms capable of synthesizing multiplexed spatial data with routine clinical pathology could democratize access to these advanced insights. Future efforts must prioritize the validation of spatial signatures in prospective clinical trials to establish their utility in guiding immunotherapy and targeted treatments. Ultimately,

bridging the gap between spatial discovery and clinical implementation will be essential to achieving precision medicine for HNSCC patients.

List of abbreviations

HNSCC: Head and neck squamous cell carcinoma

TME: Tumor microenvironment

TLS: Tertiary lymphoid structures

CAF: Cancer-associated fibroblast

ECM: Extracellular matrix

mIF: Multiplex immunofluorescence

IMC: Imaging Mass Cytometry

PCF: PhenoCycler-Fusion

TC: Tumor core

EMT: Epithelial-mesenchymal transition

pEMT: partial EMT

Tregs: Regulatory T cells

TIME: Tumor immune microenvironment

myCAFs: myofibroblastic CAFs

iCAFs: inflammatory CAFs

apCAFs: antigen-presenting CAFs

LNM: Lymph node metastasis

OSCC: Oral squamous cell carcinoma

ICIs: Immune checkpoint inhibitors

FFPE: Formalin-fixed paraffin-embedded

VEGFA: Vascular endothelial growth factor A

ANGPTL: Angiopoietin-like protein

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This manuscript is written with the help of the AI language model "ChatGPT" developed by OpenAI. The AI is only used to improve the language expression and clarity of the manuscript; the AI does not generate or create any content, including the main text, tables and figures of the manuscript. All original opinions and data belong to all authors. Authors take full responsibility of the manuscript's content.

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